Research Article

Title: Bacterial microcompartment-directed polyphosphate kinase promotes stable polyphosphate accumulation in *E. coli*

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Abbreviations: BMC, bacterial microcompartment; enhanced biological phosphorus removal , EBPR; PPK1, polyphosphate kinase; PPX, exopolyphosphatase.

Abstract

Temporary manipulation of bacterial polyphosphate levels by phased environmental stimuli underlies wastewater biological phosphate removal processes. In *E. coli* polyphosphate levels are controlled via polyphosphate kinase (PPK1, synthesizing) and exopolyphosphatases (PPX and GPPA), and are temporarily enhanced by PPK1 overexpression and reduced by PPX overexpression. We hypothesised that partitioning PPK1 from cytoplasmic exopolyphosphatases would increase and stabilise *E. coli* polyphosphate levels. Partitioning was achieved by coexpression of *E. coli* PPK1 fused with a microcompartment-targeting sequence and an artificial operon of *Citrobacter freundii* bacterial microcompartment genes. Encapsulation of targeted PPK1 resulted in stably increased cellular polyphosphate and persistent net phosphate cellular uptake into stationary phase, while temporary polyphosphate increase and phosphate uptake was observed with PPK1 overexpression alone. Targeted PPK1 increased polyphosphate in the microcompartment fraction 8-fold compared with non-targeted PPK1. Co-expression of PPX and non-targeted PPK1 abolished any increase in cellular polyphosphate from PPK1 expression alone. Co-expression of PPX with targeted PPK1 however resulted in elevated polyphosphate levels due to persisting polyphosphate in bacterial microcompartments. Subcellular polymerising enzyme targeting to bacterial microcompartments sequesters metabolic products from competing catabolism by preventing catabolic enzyme access. Specific application of this process to polyphosphate is of potential application for biological phosphate removal.

1. Introduction

Polyphosphate is a molecule thought to be present in all organisms [1] playing a role in cellular metabolic processes, stress response processes, virus replication and cell structure [2]. Bacterial polyphosphate accumulation underlies the enhanced biological phosphorus removal (EBPR) process, which uses microorganisms to remove inorganic phosphate (Pi) from wastewater [3]. Phosphate recovery processes are required to reduce eutrophication, the overgrowth of cyanobacteria and plants in water polluted by excess phosphorus from human activity [4], and because of the unsustainability of current phosphorus resources beyond the next century [5]. In EBPR, cycling of wastewater sludge through aerobic and anaerobic phases of incubation lasting several hours, when continued over a period of weeks selects a bacterial consortium that has a net effect of removing phosphorus from wastewater over the cycle by accumulating it in the sludge. Polyphosphate-accumulating bacteria are key consortium components [6, 7]. Phosphate release from the consortium occurs in the anaerobic phase in parallel with consumption of volatile fatty acids, polyhydroxyalkanoate polymer formation and glycogen utilisation. In the aerobic phase stored polyhydroxyalkanoate is catabolised, glycogen replenished and phosphate taken up to form polyphosphate granules [7]. EBPR is a complex dynamic process - an uncultured bacterium *Candidatus Accumulibacter phosphatis* performs a key role in EBPR polyphosphate accumulation [8], and an established EBPR reactor may fail for unknown reasons [3, 9].

Genetic manipulation of aspects of phosphate binding, uptake and storage by a single model organism such as *E. coli* has been suggested as an alternative or additive approach

to biological phosphorus removal [10–13]. One issue with over-expression of a polyphosphate forming enzyme in *E. coli* has been that most of the consequent increase of polyphosphate is temporary, probably because of the existence of competing catabolic enzymes [14, 15] and phosphate release from the cells then occurs as the polyphosphate is broken down.

In *E. coli* polyphosphate kinase PPK1 (E.C. 2.7.4.1) is the enzyme responsible for assembling inorganic polyphosphate polymers in the bacterial cytoplasm by catalysing the reaction nATP ⇄ (polyphosphate)n + nADP [16, 17]. Although this is a reversible reaction, in *E.coli* this enzyme generally favours synthesis of polyphosphate over breakdown (Vmax ratio of 4.1) [17] (Fig.1A). However, the balance between net accumulation and breakdown changes dynamically during culture growth and also in response to external stimuli, in part due to the action of degradative exopolyphosphatases. In this respect *E. coli* contains two such polyphosphatases that release orthophosphate from the termini of long chain polyphosphate: (polyphosphate)n → (polyphosphate)n-l + Pi. The two polyphosphatases are called PPX (E.C. 3.6.1.11, sometimes referred to as PPX1) [18, 19], which is encoded in the same operon as PPK1, and its homologue guanosine pentaphosphate phosphohydrolase (GPPA or PPX2) [17, 20]. GPPA (E.C. 3.6.1.40) also hydrolyses guanosine pentaphosphate (pppGpp) to guanosine tetraphosphate (ppGpp) with phosphate release as part of the control of the stringent response. Both PPX and GPPA are competitively inhibited by pppGpp [21]. Consequently, amino acid starvation in *E. coli* leads to the accumulation of large amounts of polyphosphate due to the high levels of pppGpp produced as part of the stringent response [21]. In *E. coli ppk1* and *ppx* are adjacent

genes forming an operon and knockout of *ppx* alone has been engineered by combined knockout of *ppk1* and *ppx* with heterologous plasmid expression of *ppk1* [15, 22, 23]. Elevation of polyphosphate levels in these cells rapidly declines a few hours after *ppk1* plasmid induction whether *ppx* is active or knocked out [15], showing that PPX is not the sole cause of instability in polyphosphate levels in *E. coli*. We hypothesized that an alternative approach to prevent the access of all other cytoplasmic enzymes, (not just PPX) to polyphosphate formed from recombinant PPK1 would stabilise cellular polyphosphate levels and create a phosphate-retaining phenotype. The mechanism used to achieve this is targeting of PPK1 to a recombinant bacterial microcompartment.

Bacterial microcompartments (BMCs) are proteinaceous vesicles found in certain bacteria that house specific metabolic pathways encased within a closed polyhedral shell of 100-150 nm diameter. The shells are made of thin protein sheets [24] containing pores less than 1 nm in diameter [25] which can be positively or negatively charged. There are two broad groups of BMCs, those associated with the anabolic process of RuBisCO-mediated carbon fixation (carboxysomes) and those associated with catabolic fermentative processes such as 1,2-propanediol utilisation (metabolosomes) [26, 27]. Although BMCs were first seen over fifty years ago in photosynthetic cyanobacteria [28], their presence in the cytoplasm of heterotrophic bacteria was only confirmed in 1998 [29] after they were detected in thin sections of *Salmonella enterica* grown on 1,2-propanediol. In fact, around twenty per cent of bacterial genome sequences contain BMC structural genes [26], in many cases associated with enzymes of unknown function [30].

A significant proportion of bacteria therefore make a major investment in retaining and expressing large (15+ gene) operons encoding these structures and associated enzymes. It is believed the structures help mediate metabolic efficiency by selective limitation of the shell pores on the passage of substrates [31], by metabolite channelling, or other mechanisms achieving retention of reaction intermediates within the structure [32, 33]. *Salmonella enterica* Serovar Typhimurium accrues a competitive metabolic advantage by BMC-mediated respiration of ethanolamine in a mouse colitis model [34]. Enterohaemorrhagic *E. coli* obtains a similar competitive advantage from BMC-mediated ethanolamine fermentation in bovine intestinal fluid [35], favouring persistent intestinal carriage.

Recombinant BMCs using genes from *Citrobacter freundii* can be expressed heterologously in *E. coli* [36], both with and without [37] the associated interior enzymes. Peptide sequences enabling enzyme localisation to the BMC interior have been identified [37] [38]. For instance, the first 18 amino acids of PduP, P18, or the first 18 or 60 amino acids of PduD (D18 or D60) can be used as fusions to direct “foreign” proteins into the BMC [39]. Compartmentalisation of the cellular interior is a functionally transforming process often thought of as characteristic of eukaryotes [40], but specific localisation of any enzyme to a re-engineered BMC in bacteria could increase metabolic flexibility of the bacterial host enabling novel phenotypes [41]. Nanotechnological applications of other biological compartment systems have included the use of viral capsids for DNA delivery [42], lumazine synthase enclosure of HIV protease [43], and the engineering of novel metabolites. We hypothesized that directing PPK to a BMC would enhance polyphosphate

formation within a cellular compartment and that segregation from the known degradative polyphosphatases and other cytoplasmic enzymes should stabilise accumulation of polyphosphate (Fig.1).

2. Materials and Methods

Strains, plasmids and culture conditions

E. coli was grown in LB or MOPS medium [44] with either 0.5 mM or 1.0 mM K_2HPO4 as indicated. Expression of pET cloned genes was induced by IPTG in *E. coli* BL21 (DE3) and *E. coli* BL21 Tuner (DE3) (Novagen). Strains and plasmids are listed in Table 1 and oligonucleotides in Supplementary Data Table S1. Incubation for phosphate uptake experiments was at 37 °C, otherwise pDuet inductions were incubated at 18 °C.

Molecular techniques

Plasmid cloning was carried out in *E. coli* JM109 or *E. coli* TOP10 (Invitrogen) with subsequent transfer to *E. coli* BL21 (DE3) and *E. coli* BL21 Tuner (DE3) for expression using standard transformation techniques [45]. For PCR experiments standard protocols were applied using an MJ Research PTC-200 Thermal Cycler for reaction cycles. Genomic DNA was extracted from *E. coli* JM109 using a Wizard® Genomic DNA Purification Kit (Promega). Plasmid constructs were sequenced commercially (GATC Biotech).

Targeting of PPK1

The strategy used is summarised in Supplementary Data Fig.S1. The *ppk1* gene coding for polyphosphate kinase (PPK1) was PCR-amplified with a proofreading DNA polymerase

(Bioline High Velocity Polymerase, Bioline UK, London), using genomic DNA from *E. coli* JM109 as template, using the forward primer ppk1-F and a reverse primer ppk1-R (Table S1). The PCR product was digested with *Sac* I and *Hind* III (Fermentas) followed by ligation to pET23b-GFP-pduP18 digested with *Sac* I and *Hind* III. The gene encoding the GFP was thus replaced by *ppk* with retention of the *pdu* localization sequence. The ligation product was transformed into *E. coli* Top 10 electrocompetent cells (Invitrogen) by electroporation. The new vector, named pML001 (pET23b*pduP18-ppk1*), was extracted and the *ppk1* insert was sequenced (GATC-Biotech) to confirm no mutation had occurred. Two constructs, pML001 and pLysS*pduABJKNU* (pSF37), expressing an empty *pdu* BMC [37]), were co-transformed into *E. coli* BL21 (DE3) by heat shock.

Co-localisation of PPK1 and Microcompartments

Peptide fingerprinting of microcompartment cellular fraction

Recombinant BMCs were extracted from *E. coli* by a modification of a published method [46]. A single colony pick was grown in 200 ml of LB to an OD of 0.4 followed by induction with 0.4 mM IPTG. Cells at OD1.0-1.2 were harvested and washed twice with 40 ml of buffer A (50 mM Tris-HCl (pH 8.0), 500 mM KCl, 12.5 mM MgCl2, 1.5% 1,2-PD). Cells (1 g wet weight) were resuspended in a mixture of 10 ml of buffer A and 15 ml of BPER-II supplemented with 5mM mercaptoethanol, Complete Protease Inhibitor Cocktail (Roche) at the manufacturer's recommended working dilution, 25 mg of lysozyme, and 2 mg of DNase I. The suspension was incubated for 30 min on a shaking incubator at room temperature and on ice for 5 min. After initial removal of cell debris by

centrifugation at 12,000 *g* for 5 min at 4°C repeated twice, the BMC fraction was precipitated by spinning at 20,000 *g* for 20 min at 4°C. The pellet was washed once with a mixture of 4 ml of buffer A and 6 ml of BPER-II and resuspended in 0.5 ml of buffer B (50mMTris-HCl pH 8.0, 50 mM KCl, 5 mM MgCl2, 1% 1,2-PD) containing Complete Protease Inhibitor Cocktail (Roche) at the manufacturer's recommended working dilution. Remaining cell debris was removed by centrifugation for 1 min at 12,000 *g* 4°C repeated three times. Aliquots (50 □g) of extracted protein were separated by SDS-PAGE using a 15% polyacrylamide gel under denaturing conditions in a MiniProtean apparatus (Bio-Rad) and stained with Coomassie Brilliant Blue R250 (Fig.1B). Peptide fingerprinting carried out as previously described [47]. In microcompartment extraction for ATP regeneration assays (Fig. 1C,D) and whole cell polyphosphate assays (Fig 2) CelLytic B (Sigma-Aldrich) was initially substituted for BPER-II because of published efficacy of this reagent for polyphosphate extraction [48]. In later microcompartment extractions (Fig. 3) comparisons of BPER-II extractions and CellLytic B extractions had shown little difference in measured polyphosphate levels and BPER-II was used. Micrococcal nuclease 2 mg (Sigma-Aldrich) prepared with calcium buffer was substituted for DNase 1 for all microcompartment extractions where polyphosphate was assayed because of the potential adverse effect of Mg^{2+} containing buffers on polyphosphate [49].

ATP regeneration assay

A combination of two previously described PPK1 assay methods [48, 50] using luciferase to detect ATP produced from polyphosphate by PPK1 was used as a biochemical screen for the presence of PPK1 and polyphosphate in microcompartment fractions. Briefly, to assay relative PPK1 content 20 □L of BMC extractwas added to a 100 □L reaction mixture

containing: ultrapure ADP (ATP-free, Cell Technology Inc, Ca), 30 mM $MgCl_2$, 1% (w/v) Polyphosphate (Sigma), 50 mM Tris-HCl (pH 7.8). The reaction mixture was diluted 1:100 in 100 mM Tris-HCl (pH 8.0)–4 mM EDTA, of which 0.1 mL was added to 0.1 mL of luciferase reaction mixture from ATP Bioluminescence Assay Kit CLS II (Roche). Luminescence was measured by using a luminometer (Luminoskan, Thermo Labsystems). A standard curve for ATP by dilution in 100 mM Tris-HCl (pH 8.0) containing 4 mM EDTA was used. To assay relative polyphosphate content, the same reaction omitting added polyphosphate was performed (Figure 1C).

Co-expression of targeted and untargeted PPK1 and PPX

The pCOLADuet-1 coexpression vector (Novagen) system encoding two multiple cloning sites (MCS) each preceded by a T7 promoter, *lac* operator, and ribosome binding site was used to express targeted and untargeted *E. coli* PPK1 and PPX (*ppx* amplified from *E. coli* JM109) in combination (pYY005, pYY007, pYY008) and alone (pYY002, pYY010) (see Table 1).

Polyphosphate concentration presented in Fig. 3 was determined following lysis of pelleted cells from 10 ml of cultures described above. A metachromatic assay was employed using the 530/630nm absorbance ratio of 10 μL of lysate added to 1 mL of toluidine dye solution (6 mg/L toluidine blue in 40 mM acetic acid) as described [51]. In later experiments (Fig. 4) polyphosphate was determined by a higher-yielding method using 4'-6-diamidino-2-phenylindole (DAPI) as described [52] on whole cells or microcompartment cell fractions obtained with BPER-II extraction. Briefly, cells were harvested by centrifuging at 5000g for 10 min at 4° C. After washing in 50mM HEPES buffer (pH7.5) the cell pellet or

microcompartment fraction was frozen at -20° C followed by defrosting at room temperature. Cell pellets/microcompartment fractions were resuspended in HEPES buffer at an appropriate dilution to ensure that the cellular polyP concentration was in the linear range of the DAPI assay (0-6 □g polyP/ml). Total assay volume was 300µl which included 100 µL of polyP containing samples and 200 µL of DAPI assay buffer containing 150 mM KCl, 20 mM HEPES-KOH (pH 7.0) and 10 µM DAPI solution. After a 10 min incubation at room temperature DAPI fluorescence was measured with a platereader equipped with excitation and emission filters of 420 nm and 550 nm respectively.

A polyphosphate standard curve was prepared using sodium phosphate glass Type 45 (S4379 Aldrich) and sodium hexametaphosphate (SX0583). Protein concentration of cell extracts was measured using a 10µL sample, with Coomassie Plus Protein Assay Reagent (Pierce) with bovine serum albumin as the standard resuspended in the same buffer as the sample.

Phosphate uptake was determined as follows (Fig. 2A). Bacteria was grown to OD_{600} 0.4-0.6 in Luria broth and then induced by 0.5 mM of IPTG for 1 hr before transfer to pH 5.5 MOPS medium [44] containing 0.01 mM iron and 0.5 mM potassium phosphate, at an OD600 of 0.2. Incubation was continued at 37°C with intermittent sampling of 0.2 mL up to 48 hrs. Samples were centrifuged and supernatant used for phosphate assay, and the pellet used for polyphosphate and protein assays. Phosphate was assayed using a molybdovanadate colorimetric method [53]. 0.2 mL of molybdovanadate solution (Reagecon, cat no: 1056700) was added to 5 mL of culture supernatant, mixed and

incubated at room temperature for 5 min. Optical density of 1 mL at 430 nm was measured against a blank of 4% molybdovanadate in distilled water and a calibration curve of potassium phosphate in MOPS.

Light Microscopy

Polyphosphate granules were visualised in fixed films (Fig. 4) by Neisser's stain using Chrysoidin counterstain [54].

Electron Microscopy

E. coli BL21(DE3) cells containing targeted/untargeted PPK1 and pLySsPduABJKNU (for expressing empty microcompartments) were grown in 50 mL of LB broth containing 100 mg/litre ampicillin and 32 mg/L chloramphenicol with shaking at 37 °C. Upon reaching an OD_{600} of 0.5, protein production was induced with 0.5 mM isopropyl-D-thiogalactoside, and the cultures were incubated by shaking overnight at 18 °C. Harvested cells were resuspended in 2 mL of fixative consisting of 2.5% glutaraldehyde in 100 mM sodium cacodylate (CAB) buffer (pH 7.2). The cells were pelleted and washed twice with CAB to remove traces of the fixing solution. Cells were then stained for 1 hr in 1% osmium tetroxide (w/v) and washed with CAB before dehydration. Dehydration was carried out by placing the samples into an ethanol gradient: 50%, 70%, 90% once for 10 minutes, and 100% dried ethanol three times for 15 minutes. Samples were rinsed twice for 15 minutes in propylene oxide and then incubated in 50/50 propylene oxide/Agar LV resin for 30 minutes. Samples were incubated 2 x 2 hr in fresh Agar LV resin before embedding in Beem capsules by centrifugation at 11,000 rpm for 5 minutes followed by incubation at 60

°C overnight to polymerize. Specimens were thin sectioned with a diamond knife on an RMC MT-6000-XL ultramicrotome, collected on 400 mesh copper grids, and post-stained with 4.5% uranyl acetate for 45 min at RT and lead citrate for 7 min at RT. Sections were then observed and photographed with a JEOL-1230 transmission electron microscope at an accelerating voltage of 80 kV.

Electron microscopy for parallel electron energy loss spectroscopy (PEELS) and element mapping by electron spectroscopic imaging (ESI)

Unstained cells were fixed in 3% (v/v) glutaraldehyde – 10 mM Hepes, pH 7.3 (Sigma), dehydrated in an acetone-series and embedded in epoxy resin (Spurr, hard mixture; [55]), as described [56]. For elemental analysis 30 - 40 nm ultrathin sections (otherwise 90 nm for general ultrastructure) were sectioned with a Reichelt-Jung ultramicrotome (Leica, Vienna, Austria), equipped with a diamond knife and were picked up with 300 mesh Cu-grids. Electron micrographs were recorded in the elastic brightfield mode (slit width: 10 eV) with an EF-TEM (operated in general at 120 kV acceleration voltage), equipped with an in-column Omega-type energy filter (LIBRA120 plus, Zeiss, Oberkochen Germany), in a magnification range from x 4000 to x 32000 with a bottom-mount cooled 2048 x 2048 CCD camera (sharp:eye; Tröndle, Moorenweis, Germany).

Parallel electron energy loss spectroscopy (PEELS)

Spot-PEELS were recorded within electron dense cytoplasmic inclusion bodies. Spot-size was set to 16 nm and the objective aperture was 60 μm (spectrum magnification: x100; energy range: 67 – 290 eV; recording time: 10 s ; emission current: 1 μA) and the spectrum

energy resolution was about 1.6 eV at zero-loss (FWHM). Recorded PEELS data were corrected for background, applying the 'potence' underground function of the EsiVision Pro Software (EsiVision Pro, Vers. 3.2; SIS – Soft Imaging Systems, Munster, Germany) and were 'medium'-filtered (settings: 1.5 eV width).

Element mapping by electron spectroscopic imaging (ESI)

Phosphorus mapping was performed as previously described [56] with unstained 35 nm ultrathin sections. According to the '3-window method' energy-windows were set to a dedicated energy loss for the P-L23 edge, as it was given by the corresponding first intensity maximum from the spot-PEELS, i.e. 138 eV (W1: 125 eV; W2: 115 eV). The energy selective slit was set to 6 eV width, and images were recorded with an illumination aperture of 0.63 mrad, an emission current of 1 μA, a 60 μm objective aperture, and a nominal magnification of x 6300. Background subtraction for calculating the phosphorus element map was performed by the 'multiwindow exponential difference' method.

3. Results

BMC localisation of PPK1

The localisation of PPK1 to a recombinant BMC was achieved by engineering the fusion of the P18 targeting peptide to the N-terminus of the enzyme. The recombinant BMC with the associated P18-PPK1 was isolated after lysis of the cells using a protein extraction reagent followed by differential salt precipitation and centrifugation. Analysis of the purified BMC fraction by SDS-PAGE revealed the presence of P18-PPK1 together with the BMC-associated shell proteins (Fig. 1B).

A functional assay designed to maximise PPK1's ATP breakdown function was employed to determine the activity of PPK1 when it was directed to the BMC. In comparison to BMC extracts from cells producing only empty BMCs or empty BMCs and non-targeted PPK, the purified BMC cell fractions from the cells co-producing BMCs and P18-PPK1 generated over twenty-fold more ATP per mg of protein from added polyphosphate (Fig.1C). There was little activity in the equivalent protein fraction that had been prepared from cells producing only P18-PPK1 (i.e. P18-PPK1 produced in the absence of BMCs). This showed that polyphosphate kinase activity had been transferred to the microcompartments by enzyme targeting.

The same ATP regeneration assay was run again but this time in the absence of any added exogenous polyphosphate (Fig.1D). Any ATP generated in this assay would therefore reflect the amount of endogenous polyphosphate within the fraction. The BMC fraction from the cells that co-produced both the BMCs and P18-PPK1 generated more than twice as much ATP as control BMC fractions from cells expressing empty BMCs or BMCs with non-targeted PPK1 (Fig. 1D). This result indicates that the BMC fraction from cells co-expressing targeted PPK1 had increased levels of polyphosphate, compatible with localisation of PPK1 to the microcompartment and formation of polyphosphate in situ.

PPK1 targeting effect on polyphosphate content and phosphate uptake,

The effect of PPK1 overexpression on cellular polyphosphate was both qualitative and quantitative, and targeted PPK1 with co-expressed BMCs gave a distinct phenotype. DAPI

negative staining of polyphosphate extracts, size-separated on a PAGE gel (Supplementary Data Figure S2), showed that the polyphosphate detected in strains over-expressing either *p18ppk1* alone or *p18ppk1* and *pduABJKNU* exceeded the length of the sodium phosphate glass Type 45 polyphosphate control. This indicates that long chain polyphosphate is present in these strains. No qualitative difference in chain length was detected between these two clones but long chain polyphosphate in the *E. coli* strain over expressing *p18ppk1* and recombinant BMCs persisted to a later phase of growth (Fig. S2) than in cells expressing *p18ppk1* alone. No long chain polyphosphate was detected in the *E. coli* control.

A simultaneous assay of the cellular polyphosphate and phosphate content of the culture supernatant from the cultures used in the polyphosphate chain length assay was also undertaken. Here, increased phosphate uptake from culture medium was observed in comparison to the host *E. coli* control (Fig. 2A) by both the *p18ppk1*-expressing strain and the strain expressing both *p18ppk1* and *pduABJKNU*. A maximal uptake of approximately 0.25 mM at 20 hours was observed for both constructs. However, the *p18ppk*-expressing strain returned a third of this phosphate to the supernatant after 48 hours, while the strain expressing both *p18ppk1* and *pduABJKNU* returned less than 9% of phosphate taken by 48 hours. Correspondingly, the cell associated polyphosphate levels of the *p18ppk1* clone were maximal at 20 hours and declined thereafter, while the *p18ppk1* and *pduABJKNU* expressing strain retained approximately the same level of cell associated polyphosphate at 48 hours as at 20 hours.

BMC protects endogenous polyphosphate from exogenous polyphosphatases

The induction of non-targeted PPK1 from the pDuet vector increased whole cell polyphosphate levels 5-fold in comparison to control cells containing the BMC shell protein operon and the pDuet vector with no enzyme insert (the enzyme-free control, Fig. 3). It did not increase the polyphosphate content of co-expressed recombinant microcompartments when compared to the enzyme-free control. However, P18-PPK1, when co-produced with the BMCs, increased polyphosphate levels in the BMC fraction 8-fold in comparison to the enzyme-free control, while giving a similar overall 5-fold increase in whole cell polyphosphate to that seen with expression of non-targeted PPK1.

Co-expression of non-targeted polyphosphatase PPX with non-targeted PPK1 reduced whole cell polyphosphate levels by 50% compared with non-targeted PPK1 expression alone, with little effect on polyphosphate levels in the microcompartment fraction. Co-expression of non-targeted PPX and BMC-targeted P18-PPK1 reduced whole cell polyphosphate levels by 22% and BMC-associated polyphosphate by 18% when compared with microcompartment targeted PPK1 alone. BMC-associated polyphosphate was still at least 2.5 times greater than in cells co-expressing non-targeted PPK1 in the presence or absence of non-targeted PPX. Co-expression of BMC targeted P18-PPK1 with PPX targeted to the microcompartment using a different tag (D60) reduced the BMC-associated polyphosphate content by 50% in comparison to the BMC-targeted P18-PPK1 alone, while reducing whole cell polyphosphate by 22%. These data suggest that the BMC-targeting of PPK1 results in the synthesis of polyphosphate that is located primarily within the BMC fraction of the cell and is relatively inaccessible to cytoplasmic co-expressed PPX, but more accessible to BMC-targeted PPX.

Microscopy

Blue-black granules were apparent with Neisser's stain in a proportion of all cells overexpressing P18-PPK1, but not the *E. coli* BL21 insert-free control or without any targeted enzyme (Fig. 4). These appearances are consistent with the accumulation of intracellular polyphosphate in *E. coli* cells with increased PPK1 activity. All cells overexpressing P18-PPK1 showed a heterogeneous granule phenotype, with a proportion of non-toluidine blue staining cells in all fields.

E. coli expressing the recombinant microcompartment and P18-PPK1 retained the polyphosphate staining at 44 hours (Fig. 4) whereas cells expressing P18-PPK1 without the recombinant microcompartment showed reduced staining after 40 hours (Fig. 3).
All *E.coli* expressing the recombinant microcompartment had a proportion of cells which were greatly elongated. All *E.coli* forming multiple polyphosphate granules tended to be larger than the non-granulated cells, presumably because of distension by the granules. However, the largest cells were seen with the combination of recombinant microcompartment and P18-PPK1.

Electron-loss spectroscopic analysis by Energy-filtered Transmission Electron Microscopy (EFTEM).

Increased phosphorus deposition was detected in all cells expressing recombinant *E.coli* PPK1 (Fig. 5C,D,E,F), verified from PEELS measurement (see below), compared with

control *E. coli* strains with no recombinant gene expression (Fig. 5A) or expressing microcompartment genes (Fig. 5B). In cells expressing PPK1 alone, most phosphate signal was represented by particles <5 nm, but some large homogeneous masses > 200 nm with plane edges were visible (Fig. 5C) in a few cells. In cells expressing targeted PPK1 and a recombinant microcompartment operon, in addition to signals from particles <5 nm, multiple phosphate signals from particles 50-100 nm were present (Fig.5D,E,F) and in some cases large circular masses/crescents > 300 nm were present (Fig. 5D,F). These large masses were not homogeneous and appeared composed of small particles and the cells containing them were enlarged. These images appeared similar to light microscopy observations (Fig. 4D,H,L).

Parallel electron energy loss spectroscopy (PEELS)

Spot-PEELS recorded from dark inclusions apparent as electron dense regions about 100 nm in diameter (Fig 5G), confirmed they contained phosphate, verified from the characteristic ELNES-fingerprint (Energy-Loss Near-Edge Structure) of reference spectra that were recorded from sodium polyphosphate (Fig.5G). The largest polyphosphate inclusion in figure 5E, shown in yellow, is magnified in the inset of the spot-PEELS (Fig. 5G); here the 16 nm beam spot and its position are indicated (white circle).

4. Discussion

Polyphosphate accumulation is the basis of the enhanced biological phosphorus removal (EBPR) process, which uses microorganisms to remove inorganic phosphate (Pi) from wastewater. Accumulation occurs in aerobic conditions as intracellular polyphosphate [8, 12] is released as Pi in anaerobic conditions [57] and supplied with organic carbon or heated [12]. The best characterized enzyme responsible for polyphosphate synthesis (PPK1), originally found in *E. coli* [58], can only be detected by bioinformatics in the genome sequences of a minority of bacterial genera [59]. The enzyme responsible for polyphosphate synthesis in most bacteria therefore remains to be identified [59].

In *E. coli*, polyphosphate accumulation in wild-type strains occurs with amino acid starvation or in the stationary phase [21, 50, 60]. Large amounts of polyphosphate accumulate only if the copy number of *ppk* is increased, or a heterologous *ppk* gene is supplied, or *phoU* is mutated [61]. Even in *E. coli* strains overexpressing *ppk*, initial accumulation of polyphosphate is known to be partially or completely reversed as the cells reach stationary phase [14, 15]. Because this also occurs in *E. coli* overexpressing *ppk* with no chromosomal functioning *ppx* gene it has been suggested to be due to either product-induced reversal of the PPK-catalysed reaction, or the activity of another phosphatase enzyme present in the cytoplasm [15]. We observed a similar reversal of polyphosphate accumulation in our overexpressing *ppk* clone, accompanied by increasing Pi in the culture supernatant (Fig. 2). This did not occur when the *ppk* gene was engineered to encode an N-terminal BMC localisation sequence and was expressed in trans with an operon encoding for an empty BMC.

Cells co-producing P18-PPK1 and the empty BMC had a different phosphorus distribution by EFTEM (Fig 5D,E,F) to those expressing PPK1 alone (Fig. 5C), containing single or agglomerated particles in the BMC size range. BMC extractions show the presence of metabolically active PPK1 (Fig.1C,D) and polyphosphate in the BMC fraction (Fig.1D,Fig.3) when PPK1 is microcompartment-targeted in this way. Our results suggest that targeting of PPK1 to a bacterial microcompartment still allows access of the small molecule substrate ATP to the enzyme (Figure 1A), but effectively stabilises the large polymer polyphosphate product (Fig. 2B,3).

We hypothesized that this stabilisation results from reduced access of PPX, GPPA or other cytoplasmic phosphatases to the polyphosphate produced by BMC-targeted PPK1. To confirm this we carried out co-expression experiments of PPK1 with PPX (Fig. 3). Co-production of PPX with PPK1 resulted in lower cellular polyphosphate levels than expression of *ppk1* alone (Fig. 3), as has been previously reported [62]. This reduction in total cellular polyphosphate was partially prevented by BMC-association of PPK1, due to increased levels of polyphosphate in the BMC fraction. BMC-targeting of PPK1 therefore results in the synthesis of polyphosphate that is located primarily in the BMC fraction of the cell. Polyphosphate in the BMC fraction is inaccessible to cytoplasmic co-expressed PPX. Adding BMC targeting to PPX (D60-PPX) co-expressed with targeted PPK1 (P18-PPK1) partially reverses the increase in polyphosphate levels in the BMC fraction conferred by targeted PPK1, presumably by increasing access of the PPX to polyphosphate in the BMC fraction. This suggests that the mechanism of stabilisation of

polyphosphate conferred by BMC targeting of PPK1 involves reduced access by cytoplasmic phosphatases.

Other examples of such macromolecular association of enzymes exist. A variant of lumazine synthase was recently employed to encapsidate HIV protease within an *E. coli* host [43] facilitating recombinant synthesis of this potentially toxic enzyme by separating it from the remaining cytoplasm. Lumazine synthase compartments are genetically unrelated to BMCs involved in catabolic metabolism, and form pentameric components form smaller 30-40 nm icosahedral structures that more closely resemble viral capsids [63]. The enzyme is bound to part of the shell molecule forming the inner surface by an electrostatic mechanism [43, 64], (N-terminal fusion displays it on the outside [65]). Enzymically active inclusion bodies can be formed within bacterial cells by C-terminal attachment of short self-assembling peptide sequences [66], or N-terminal fusion with a self aggregrating protein [67] but these enzymes are not enclosed within a structure accessed via pores. Subcellular localisation of enzymes catalysing successive reactions in a metabolic pathway to peroxisomes in fungi [68] or BMCs [39] can promote product formation.

Our results demonstrate that P18-PPK1 is targeted to a recombinant BMC. The observation that polyphosphate accumulates within the BMC suggests that targeted PPK1 is internalised within the structure and remains functional, generating polymeric product. ATP must be able to enter the recombinant BMC to allow it to act as one of the substrates for the P18-PPK1 enzyme (Fig. 1A). However, this is not surprising as the native Pdu

BMC must allow ATP access as it is required by PduO (located within the microcompartment) for the regeneration of the coenzyme form of cobalamin needed by the diol dehydratase complex [69]. The association of PPK1 with the BMC however leads to sequestration of the enzyme's metabolic product, presumably because its size does not allow it to leave the BMC by the same route by which the enzyme substrate ATP arrived. Protection of the polyphosphate product from catabolism from cytosolic enzymes is therefore achieved, illustrating a general mechanism by which BMC can be used to re-engineer cellular metabolism. The specific polymer generated, polyphosphate, is an important intermediary in the enhanced biological phosphate removal (EBPR) process employing environmental bacteria to remove phosphate from wastewater [3, 12] and has industrial applications [70]. EBPR requires prolonged cycles of aerobic and aerobic incubation to operate. The ability to stabilise polyphosphate produced in a single growth phase so that phosphate is not returned to the cell exterior could lead to a streamlined process with a single phase of incubation. This would require transfer of the recombinant microcompartment and targeted enzyme from *E. coli* to a more environmentally robust organism.

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Table 1
Plasmids and strains used in this study

Plasmids and strains	**Genotype***	**Source**
Plasmids		
pET23b	pBR322, T7 Ap	Novagen
pCOLADuet-1™	ColA ori lacI T7lac Kan^r	Novagen
pET23b-GFPPduP18	pET23b with *gfp*▲ and *pduP18*† leader sequence	Prof. Martin Warren, University of Kent
pLysSPduABJKNU (pSF37)	Cam^R , Tet^R *pduABJKNU*†	[37]
pML001	pET23b with pduP18†-*ppk1* fusion without *gfp*▲	This study
pML002	pET23b-*ppk1*	This study
pCOLADuetPPK (pYY002)	pCOLADuet-1 with *ppk1*	This study
pCOLADuetP18PPK (pYY010)	pCOLADuet-1 with *pduP18*†-*ppk1* fusion	This study
pCOLADuetPPXPPK (pYY005)	pCOLADuet-1 with *ppk1* and *ppx*	This study
pCOLADuetP18PPKPPX (pYY007)	pCOLADuet-1 with *pduP18*†-*ppk1* fusion and *ppx*	This study
pCOLADuetD60PPXP18PPK (pYY008)	pCOLADuet-1 with *ppk1* and *pduD60*†-*ppx* fusion	This study
Strains		
E.coli JM109	*endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB*$^+$ *Δ(lac-proAB) e14- hsdR17(*r_K^- m_K^+*)*	Promega
E.coli Top 10	*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(*Str^R*) endA1* λ^-	Invitrogen
E.coli BL21 (DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B) *gal dcm* (DE3)	Stratagene
E.coli Tuner (DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B) *gal dcm lacY1* (DE3)	Stratagene

*All inserts from *E.coli* JM109 unless specified †From *Citrobacter freundii* ▲From *Aequorea victoria*

Figure Legends

Fig. 1. Effect of microcompartment-targeting of polyphosphate kinase (PPK1) in *E. coli*

A. Proposed mechanism of increasing polyphosphate content of *E. coli* by microcompartment-targeting of polyphosphate kinase (PPK1).

B. SDS-PAGE gel of *E. coli* BL21 (DE3) microcompartment extracts showing presence of PPK1 band. M: MW marker. NE: no enzyme, microcompartment only, (pLysSPduABJKNU). PPK1 : non-targeted PPK1 plus microcompartment, pML2 (ppk1) pLysSPduABJKNU. P18PPK1(2 lanes): microcompartment-targeted P18PPK1 fusion plus microcompartment, pML1 (p18ppk1) pLysSPduABJKNU.

C & D. ATP generation assay with microcompartment fraction of *E. coli* BL21 (DE3) as substrate detecting polyphosphate kinase activity and polyphosphate when PPK1 is microcompartment-targeted. NE, PPK1, P18PPK1: as above. NS,P18PPK1: no shell, plasmid-located targeted PPK1 only, pML1 (p18ppk1). C: polyphosphate kinase (PPK1) assay with addition of ADP and polyphosphate. D: polyphosphate assay with addition of ADP alone.

Fig. 2. Co-expression of targeted PPK1 and recombinant microcompartments in *E. coli* results in stable polyphosphate retention and orthophosphate uptake. Dashed line with filled circles: NS, *E. coli* BL21 DE3 control. Continuous red line with filled squares : NS,P18PPK1, no shell, plasmid-located targeted PPK1 only, *E.coli* BL21 DE3 pML01 (*p18ppk1*). Continuous green line with filled triangles : S,P18PPK1, microcompartment-targeted P18PPK1 fusion plus microcompartment shell *E.coli* BL21 DE3 pML1 (*p18ppk1*) pLysSPduABJKNU

A. Supernatant orthophosphate levels. B. Whole cell polyphosphate content

Fig. 3. Co-expression of microcompartment-targeted PPK1 and recombinant microcompartments in *E. coli* increases the polyphosphate content of the microcompartment fraction of lysed cells and protects it from co-expressed cytoplasmic polyphosphatase

DAPI polyphosphate assay from *E.coli* BL21 DE3 pLysSPduABJKNU all expressing recombinant microcompartments with different co-expressed enzymes. Blue bars polyphosphate content of microcompartment extractions, purple bars whole cell polyphosphate content. NE: no enzyme, microcompartment only, (pLysS*PduABJKNU*). S,PPK1 : non-targeted PPK1 and microcompartment, pML2 (*ppk1*). PPX,PPK1 : non-targeted PPK1 and non-targeted PPX, pYY005 (*ppk1 ppx*). D60PPX,P18PPK: targeted PPK1 and targeted PPK, pYY08 (*ppk1 pduD60-ppx*) . PPX,P18PPK1 : targeted PPK1 and non-targeted PPX, pYY07 (*pduP18-ppk1 ppx*). P18PPK1: targeted PPK1, pYY010 (*pduP18-ppk1*)

Fig. 4. Co-expression of targeted PPK1 and recombinant microcompartments in *E. coli* results in cytoplasmic polyphosphate granule formation persisting into stationary phase. Light microscopy of Neisser stained fixed cells (toluidine blue and chrysoidine counterstain. A,E,I: control *E. coli* BL21 DE3. B,F,J: *E. coli* BL21 DE3 NE: no enzyme, microcompartment only, (pLysS*PduABJKNU*). C,G,K *E.coli* BL21 DE3 pML01 (*pduP18-ppk1*). D,H,L BL21 DE3 pML01 (*pduP18-ppk1*) pLysS*PduABJKNU*. Incubation time in MOPS : A,B,C,D 4 hours, E,F,G,H 18 hours, I,J,K,L 44 hours.

Fig. 5. Phosphorus content of cytoplasmic granules in *E. coli* expressing recombinant polyphosphate kinase is confirmed by ultrastructural and electron-loss spectroscopic analysis using energy-filtered transmission electron microscopy (EFTEM) and is increased and qualitatively altered by recombinant microcompartment co-expression
A: control *E. coli* Tuner™(DE3). B: *E. coli* Tuner™ (DE3) NE: no enzyme, microcompartment only, (pLysS*PduABJKNU*). C: *E.coli* BL21 DE3 pML01 (*pduP18-ppk1*). D,E,F,G: *E. coli* Tuner™ (DE3) pET23bPduP18ppk1 pLySsPduABJKNU .
A-F: Electron spectroscopic imaging. Phosphorus signals are shown as overlays: green in A,B,C,F: red in D: yellow in E. Scale bar 1 □m unless stated. G:Parallel electron energy-loss spectroscopy (PEELS) of the largest granule in E. The red line represents SpotPEELS of the large inclusion from E with the spot (size: 16 nm) placed centrally (electron micrograph inset). The green-boxed area represents the P-L2,3 energy-loss near-edge structure (ELNES), characterized by the two peaks (asterisks). The blue-coloured dashed spectrum is referenced from sodium polyphosphate.